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Date deposited:

21/06/2016

Embargo release date:

18 November 2016

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Unconventional T cell recognition of an arthritogenic epitope of proteoglycan aggrecan released from degrading cartilage

Short title: Unconventional T cell recognition of degrading cartilage [56 char/spaces]

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Keywords: Rodent, cartilage, T cells, Autoimmunity, Antigens/Peptides/Epitopes, Antigen Presentation and Processing.

Abbreviations:

SUMMARY

It has been proposed that peptide epitopes bind to MHC class II molecules to form distinct structural conformers of the same MHC-II-peptide complex termed type A and type B, and that the two conformers of the same peptide-MHC-II complex are recognized by distinct CD4 T cells, termed type A and type B T cells. Both types recognise short synthetic peptides but only type A recognise endosomally processed intact antigen. Type B T cells that recognise self peptides from exogenously degraded proteins have been shown to escape negative selection during thymic development and so have the potential to contribute to the pathogenesis of autoimmunity.

We generated and characterised mouse CD4 T cells specific for an arthritogenic epitope of the candidate joint autoantigen proteoglycan aggrecan. Cloned T cell hybridomas specific for a synthetic peptide containing the aggrecan epitope showed two distinct response patterns based on whether they could recognise processed intact aggrecan. Fine mapping demonstrated that both types of T cell recognised the same core epitope. The results are consistent with the generation of aggrecan-specific type A and type B T cells.

Type B T cells were activated by supernatants released from degrading cartilage indicating the presence of antigenic extracellular peptides or fragments of aggrecan. Type B T cells could play a role in the pathogenesis of proteoglycan-induced arthritis in mice, a model for rheumatoid arthritis, by recognising extracellular peptides or protein fragments of joint autoantigens released by inflamed cartilage. [238w]

Introduction

Soon after the discovery that TCR recognize peptides bound to MHC molecules (1,2), it was shown that the local environment influences the conformation adopted when peptides bound to MHC-II (3,4). On this background, the results of a study of HEL presentation by the A^k molecule suggested that MHC-II formed complexes with a single peptide in more than one conformation distinguishable by TCR (5-8). Similar observations were made for an E^k-restricted epitope of HEL (9), and for autoantigens, binding to a number of H-2 A and E alleles and to HLA DR1 (9-16). More detailed investigation showed that a proportion of T cells from mice immunized with a synthetic HEL peptide recognized the peptide in vitro but not processed intact HEL, whereas epitope specific T cells from HEL-immunized mice recognized both synthetic peptide and processed HEL (9,17). The data were interpreted as showing the formation of two conformers, termed type A and type B peptide-MHC-II complexes, recognized by CD4 T cells termed type A and type B T cells with specificity for one or other conformer (reviewed in (18-20)). Subsequent studies have shown that peptide-MHC complex formation is influenced by the mechanism of generation of the peptide epitope, so that the type A conformers are generated after processing of intact proteins in acidic endosomal compartments of APC and loading of newly synthesized MHC-II under the control of the chaperone DM (21). Alternatively, exogenous peptides were shown to bind to mature MHC-II molecules in early endosomes or at the APC surface in the absence of functional DM resulting in formation of a mixture of type A and type B T conformers (21). Infection of mice with Salmonella typhimurium has also been shown to bias HEL presentation by dendritic cells towards a type B T cell response by a Dectin-1 dependent mechanism (22,23). Furthermore, mice expressing endogenous HEL were used to show that type B T cells escape negative selection during thymic

development *in vivo*, whereas the majority of type A T cells are deleted (24), creating a scenario in which self-reactive type B T cells could persist in peripheral lymphoid organs. Type B T cells specific for the autoepitope of insulin 9-23/A^{g7} have been cloned from pancreatic islets, the site of autoimmune damage (16), and so localised where they could play a role in autoimmunity. However, the source of natural peptide ligands that preferentially activate type B T cells in autoimmune disease has not been reported.

The major cartilage component proteoglycan aggrecan is a candidate autoantigen in inflammatory arthritis (25) and immunization of BALB/c mice with aggrecan in adjuvant induces an inflammatory arthritis mediated by CD4 T cells specific for the immunodominant arthritogenic epitope 84-103 (26). In addition, extracellular proteolysis is a characteristic of inflammatory arthritis, resulting from upregulation of matrix degrading metallo-proteinases and increased degradation of cartilage matrix components including aggrecan (27). Thus we reasoned that the T cell repertoire includes type B T cells that may recognise extracellular aggrecan fragments released during inflammation. Here we demonstrate both type A and type B patterns of recognition amongst CD4 T cell hybridomas specific for the same core epitope of aggrecan. We further show that aggrecan-specific type B T cells are activated by exogenous antigenic ligands released from degrading cartilage. The results are a step towards determining whether type B T cells participate in proteoglycan-induced arthritis in mice, and more generally whether they contribute to the pathogenicity of rheumatoid arthritis and other autoimmune disease in man.

Materials and Methods

Reagents

Proteoglycan aggrecan was purified from bovine nasal cartilage as previously described (28). The synthetic peptide ^{84}VVLLVATE**GRVRVNSAY**QDK^{103} of bovine aggrecan includes the core arthritogenic CD4 T cell epitope ^{92}GRVRVNSAY^{100} ((29) where the sequence is numbered 73-81), see the Immune Epitope Data Base epitope ID 107090, 132189 and 137285 at http://www.immuneepitope.org/. The sequence of the aggrecan epitope is identical in human (Acc. No. P16112) and bovine (Acc. No. P13608) aggrecan but differs from mouse aggrecan by two amino acids (^{92}G**Q**VRVNS**I**Y^{100,} Acc. No. Q61282). Aggrecan peptide 84-103, and the same peptide modified by substitution of citrulline for arginine at position 93 (93cit95R), 95 (93R95cit) or both (93cit95cit) compared to the native sequence (93R95R), were purchased from JPT Peptide Technologies GmbH, Berlin, Germany. Peptides for fine mapping were purchased from Proimmune (Oxford, UK), and represent staggered peptides of 14 amino acids offset by 1 amino acid inclusive of the aggrecan sequence 84-108, and peptides with alanine substituted sequentially at positions 90 to 103, except glycine substituted for the alanine at position 89.

Bovine nasal cartilage and healthy human articular cartilage explants were prepared as described previously (30). Human cartilage from the femurs of rheumatoid arthritis patients undergoing surgery were obtained with local ethical approval. Briefly, cartilage was cut into 1 mm^3 fragments and 0.5 g of fragments transferred to 5ml serum-free medium (RPMI-1640 with 2mM glutamine, 30 µg/ml gentamycin and 0.05mM 2-mercaptoethanol) in wells of a 6 well plate. Plates were incubated in a humidified environment at 37ºC with 5% CO_2. The serum free medium was changed after 24 hours and 1 ng/ml of recombinant human IL-1α and 10 ng/ml human Oncostatin-M (gifts from Dr. K. Ray, Glaxo-Smith-Kline, Stevenage) were added (30). Plates were further incubated at 37ºC for up to 20 days and supernatants were removed and stored

at -30ºC prior to use in T cell hybridoma assays.

Mice

BALB/c mice were purchased from Charles River (Margate, UK) and the *in vivo* procedures described were performed under the terms of the Animals (Scientific Procedures) Act 1986 authorized by the Home Secretary, Home Office UK. Mice were immunized in one footpad with 25 μg of aggrecan or synthetic peptide in Titermax adjuvant (Sigma-Aldrich, Gillingham, UK) and popliteal lymph nodes were removed 7 d later for ex-vivo assay.

Cells

Culture medium reagents and other chemicals were purchased from Sigma-Aldrich (Gillingham, UK), unless otherwise stated. All cells were grown in culture medium consisting of RPMI 1640 containing 3 mM L-glutamine, 50 μM 2-ME, 10% v/v FBS (BioSera, Ringmer, UK) and 30 μg/ml gentamicin.

Macrophages were grown from femoral bone marrow cells of BALB/c mice by culture in bacteriological Petri dishes for 6 days in culture medium supplemented with 1 mM sodium pyruvate, 5% horse serum and 5% of a supernatant from the L929 fibroblast cell line as a source of M-CSF as described previously (31). Cells were activated by treatment with 1 ng/ml IFN-γ (R&D Systems, Abingdon, UK) overnight at 37˚C in a humidified CO_2 incubator and shown by flow cytometry to be at least 90% positive for expression of the macrophage marker F4/80.

Dendritic cells (DC) were also grown from femoral bone marrow cells by culture for 10 days in 6-well plates in culture medium supplemented with 20 ng/ml rGM-CSF, replenishing the supplemented medium every 3 days. Cells were then incubated with

142 0.2 µg/ml lipopolysaccharide to induce maturation, and shown by flow cytometry to be
143 >80% DC for the dendritic cell marker CD11c.
144 The H-2^d expressing B lymphoma cell line A20 (TIB-208) and macrophage cell line
145 J774A.1 (TIB-67), were obtained from ATCC.
146 Stable T cell lines were derived from lymph node cells from peptide or aggrecan
147 immunized BALB/c mice by 2 to 3 rounds of restimulation with synthetic 84-103
148 peptide and irradiated spleen cells as APC, followed by expansion in rIL-2, before
149 polyethylene glycol-mediated fusion with the $TCR^{\alpha-\beta-}$ BW5147 leukaemic cell line, as
150 described previously (32). The majority of T cell hybridomas were shown to respond
151 to aggrecan peptide 84-103 and express CD4, CD3, and $TCR_{\alpha\beta}$ by flow cytometry.
152 The following T cell hybridomas were used in this study: 2G9 (Vβ8.3), 1271b (Vβ8.2),
153 1G2 (Vβ8.3), 2D7 (Vβ14).
154
155 *Lymph node proliferation assay*
156 Popliteal lymph nodes were removed 7 d after footpad immunisation of BALB/c mice.
157 Popliteal lymph node cells were cultured in the presence or absence of a range of
158 doses of aggrecan or synthetic peptides for 72 h at 3 x 10^5 per well in 96-well round
159 bottom microtiter plates. 5 KBq per well of tritiated thymidine (NET027A005MC, sp.
160 act. 0.074 TBq/mM, Perkin-Elmer, Cambridge, UK) was added for the final 16 h of
161 culture before harvesting on glass fibre membranes. Radioactivity was quantitated
162 using a liquid scintillation counter (Perkin Elmer Microbeta Trilux) and results are
163 presented as mean cpm ± SEM. Responses two-fold above medium only controls
164 were considered significant.
165
166 *T cell hybridoma assay*

Antigen presenting cells (4 x 10^4/well macrophages, A20 B cells or DC) were plated in medium with or without a range of doses of aggrecan or synthetic peptide, or diluted cartilage explant supernatants in triplicate wells and incubated for 3 h. T cell hybridoma cells (4 x 10^4/well) were added and plates were incubated for a further 24 h and then frozen. Responses of T cell hybridomas were determined as the amount of IL-2 released in a bioassay measuring proliferation of IL-2 dependent CTLL-2 cells. CTLL-2 cells (3 x 10^4/well) were incubated in triplicate wells of flat-bottomed 96-well microtiter plates in the presence of T cell hybridoma culture supernatants diluted 1:2. Plates were incubated for 24 h and labelled with tritiated thymidine and results expressed as described for the lymph node proliferation assay above. Experiments were repeated no less than three times.

Results

Lymph node proliferation responses of aggrecan and peptide immunised mice

BALB/c mice were immunised with bovine aggrecan or with the synthetic peptide representing amino acids 84-103 of aggrecan, containing the major arthritogenic CD4 T cell epitope, and proliferation to aggrecan and peptide was recalled in vitro (Fig. 1). Mice immunized with aggrecan responded to both peptide and aggrecan to a similar extent, confirming the immunodominance of the response to the arthritogenic epitope contained within peptide 84-103 (Fig. 1a). Mice immunized with peptide responded to both peptide and aggrecan, but the response to peptide was of a greater magnitude (Fig 1b), suggesting more T cells responded to peptide alone than to both peptide and aggrecan. The specificity of response for the immunizing antigens was confirmed by the lack of response in HBSS immunized mice (Fig. 1a). One interpretation of the results is that peptide-immunisation induces two populations of peptide-specific T cells

differing in their ability to recognize processed aggrecan, reminiscent of the type A and type B T cell responses reported for epitopes of HEL (18).

Patterns of recognition of T cell hybridomas to aggrecan and peptide

To investigate further we cloned and immortalised T cells from peptide 84-103 immunised mice and measured their responses to peptide and aggrecan. Of 23 84-103-specific T cell hybridomas generated from peptide-immunized mice, 9 responded to peptide and aggrecan and 14 responded to peptide only, consistent with the generation of cloned T cells with type A and type B recognition patterns. Dose response titrations for presentation of aggrecan and peptide by bone marrow-derived macrophages, dendritic cells (DC) and immortalised A20 B cells to representative T cell hybridomas are shown in Fig. 2. T cell hybridomas 2G9 (Fig. 2a,e) and 1271b (Fig. 2b,f) responded equally well to aggrecan and peptide when presented by either macrophages or DC, typical of type A T cells. T cell hybridomas 1G2 (Fig. 2c,g) and 2D7 (Fig. 2d,h) responded to peptide but not to aggrecan presented by macrophages or DC over the same antigen dose range. Although A20 B cells process and present aggrecan less efficiently, the same pattern was still observed, in that T cell hybridomas 2G9 and 1271b responded to peptide and aggrecan (figs. 2i,j), but hybridomas 1G2 and 2D7 responded to peptide only (Fig. 2k,l). All four peptide specific hybridomas clearly showed one or other of the two recognition patterns, independent of the type of APC, with no intermediate patterns of response.

Fine specificity of aggrecan epitope recognition

To establish whether the patterns of recognition could be accounted for by the presence of more than one epitope within peptide 84-103, the fine specificity of type

A and type B T cell hybridomas was determined using overlapping peptides staggered by one amino acid (Fig. 3A), as well as peptides in which each position was serially substituted with alanine (Fig. 3B). T cell hybridomas 2G9, 1271b, 1G2 and 2D7 differed in the requirement for the presence of particular amino acids of the core epitope and flanking amino acids (Fig. 3A), ranging from maximum response to 86-99 to 92-105 staggered peptides (2G9) to maximum response to only 89-102 (2D7), but responses of all four T cell hybridomas tested centred on peptide 89-102.

Fine specificity differences were also observed in the responses to serially alanine substituted peptides (Fig. 3B). A clear dependence on arginine at position 95 and asparagine at position 97 was observed, suggesting that the P4 and P6 MHC contact residues of the core epitope, defined previously as P1-P9 ^{92}GRVRVNSAY^{100} (29), is a common feature of all T cell hybridomas studied. Thus the response to staggered and substituted peptide sets showed that T cells with both the type A and type B recognition patterns focussed on a single core epitope binding to A^d in the same register.

T cell hybridoma recognition of citrullinated aggrecan peptides

Different patterns of recognition of the aggrecan epitope could result from post-translational modifications such as citrullination of one or both of the arginines within the aggrecan ^{92}GRVRVNSAY^{100} epitope as a result of the activity of peptidyl arginyl deaminases known to be present both within Rheumatoid synovium (33) and within the endosomal system of APC (34). We tested synthetic 84-103 peptides in which arginine at position 93 (93cit, P2 of the core epitope) or 95 (95cit, P4 of the core epitope), or both (93cit95cit), were substituted with citrulline, and compared recognition by type A and type B T cell hybridomas using the macrophage cell line J774A.1 as APC (Fig. 4). Both type A and type B hybridomas recognised the

unmodified peptide (93R95R) (Fig. 4). All hybridomas also recognized the peptide citrullinated at position 93, and both type A hybridomas, but neither type B, responded to the peptide citrullinated at position 95, all in the sub-micromolar dose range. None of the hybridomas recognised the doubly modified peptide 93cit95cit. The similar responses of all hybridomas to the peptide citrullinated at position 93, excludes the possibility that the different recognition patterns resulted from this post translational modification. The response of type A but not type B T cell hybridomas to the peptide citrullinated at position 95 could represent an alternative to conformational isomerism in explanation of type B recognition.

T cell hybridoma recognition of supernatants from degrading cartilage

Cartilage turnover is catalysed *in vivo* by metallo-proteinase enzymes that degrade aggrecan and other matrix components, the activity of which can be induced by the presence of inflammatory cytokines IL-1α and oncostatin-M (30). We recapitulated this milieu of degradation products *in vitro* by incubating bovine and human cartilage explants with these inflammatory cytokines and removing supernatants for use as antigen in T cell assays. Supernatants were added to antigen presentation assays with type A and type B T cell hybridomas and bone marrow macrophages as APC. Type A T cell hybridoma 1271b responded to doses of less than 1% of cartilage supernatants and responses increased proportional to the incubation time. Responses to supernatants reached the magnitude of the peptide control by 10 days of cartilage incubation (Fig. 5a), consistent with the kinetics of aggrecan release in this system (30). T cell hybridoma 1271b also responded optimally to doses of less than 1% of the three supernatants from degrading human cartilage, reaching the magnitude of the peptide control (Fig. 5b).

The two type B T cell hybridomas 1G2 and 2D7 also recognised supernatants from degrading bovine and human cartilage explants, but much less efficiently than the type A T cell hybridoma 1271b (Fig. 5a,b). Responses of both type B T cell hybridomas required doses greater than 1% of supernatant and cartilage incubation for 20 days, and were of a low magnitude compared to the peptide control (Fig. 5a). Both type B T cell hybridomas also responded to all three supernatants from degrading human cartilage (Fig. 5b). Despite the low magnitude of responses, the data suggest that aggrecan-derived type B ligands are released and available for presentation during cartilage degradation. Relatively small amounts of type B T cell ligands compared to intact aggrecan could stimulate type B T cells that have escaped thymic deletion, when type A T cells would be scarse due to their more complete deletion.

Discussion

We show here that T cell hybridomas specific for a major arthritogenic epitope of the cartilage component proteoglycan aggrecan express TCR that display recognition patterns characteristic of type A and type B T cells. This pattern has previously been defined for epitopes of HEL, cytochrome-c, E_{α}, A_{β}^{k}, myelin basic protein and insulin, and includes epitopes binding to a range of mouse MHC class II A and E alleles as well as to HLA-DR1 (8-13,15,16,18,35). Our data is the first report of type B T cells restricted by A^{d}, and the first for type B T cell recognition of a candidate joint autoantigen. The number of different antigens and MHC alleles that have been studied suggest that type B T cell recognition is a universal property of TCR-peptide-MHC class II interactions. The most likely explanation for the different patterns of recognition is that type A and type B T cells recognise distinct conformational isomers of a single peptide-MHC complex (18), a conclusion supported by biochemical data (11,36-38),

but no x-ray crystallographic evidence to date. However, a similar recognition pattern could result from a number of alternative mechanisms (discussed in (18)): (i) Contamination of peptides during synthesis or storage leading to the presence of peptides of different specificities, although we have used freshly dissolved peptides obtained from more than one commercial supplier to minimise this possibility. (ii) Free N- or C-terminal amino acids flanking the epitope in shorter synthetic peptides dictating distinct patterns of TCR fine specificity, excluded here by the observation that type B T cells recognised a number of adjacent staggered peptides with different terminal amino acids. (iii) Post-translational modification of amino acids leading to distinct TCR specificities. We investigated the response of T cell hybridomas to the aggrecan peptide 84-103 modified by citrullination at position 93, 95 or both. The responses of type A and B T cells were similar to peptides citrullinated at position 93 or at positions 93 and 95, but type A cross reacted with the peptide citrullinated at position 95 whereas type B T cells did not. This could be a result of the reduced sensitivity of type B T cell recognition but is unlikely to explain the difference in type A and type B recognition. All T cell hybridomas cross reacted between unmodified 84-103 and the peptide citrullinated at position 93, and none of the hybridomas recognised the doubly citrullinated peptide, so that citrullination could not explain the type A and type B T cell recognition patterns observed. (iv) Different populations of T cells specific for overlapping dominant and cryptic epitopes present on the same peptide, for which there are precedents (39-42), that would result in the differential recognition of intact protein by T cells against the two epitopes. The T cell hybridomas used here were shown to be specific for the same core aggrecan epitope 84-103 by fine mapping with staggered and substituted peptide sets, and only a single epitope was localised to this region in extensive epitope mapping of aggrecan in BALB/c mice (43).

As type B T cells recognise peptides but not intact protein antigens after intracellular processing, we propose that the peptide ligands required for type B T cell activation are generated by extracellular degradation during the inflammatory response. It is known that peptides in the extracellular milieu directly bind MHC class II on the APC surface (44) or in early endosomes (45-49). Treatment of DC with TLR ligands or IFN-α *in vitro* (50) or their administration *in vivo* (51) leads to presentation of epitopes from intact proteins to type B T cells probably as a result of extracellular processing, although the mechanisms were not investigated. However, a source of natural ligands that activate type B T cells has not been previously reported.

Hyaline cartilage turnover in healthy synovial joints is mediated by a family of metallo-proteases, including aggrecanases, and these enzymes contribute to the cartilage loss observed in inflammatory arthritis (27). Extracellular matrix degradation products have been identified in synovial fluid from patients with arthritis (52-54), and fragments of collagen and hyaluronan have been shown to contribute to the inflammatory response (55). These extracellular antigenic fragments are presumed to be the basis for the proposal that 'remnant epitopes' are generated from cartilage components in the extracellular space that are then presented to T cells as a mechanism that perpetuates autoimmunity (56-58). Whether it is type A or type B T cells which participate in this response has not been investigated to date. We used bovine and human cartilage explants treated with pro-inflammatory cytokines to mimic cartilage loss during inflammatory arthritis, and showed that the supernatants generated could activate aggrecan-specific type B T cells, suggesting degrading cartilage releases aggrecan peptides that are available for presentation to type B T cells. The responses were detectable only after extended periods of incubation of cartilage explants and the magnitude of response was also low compared to the responses of type A T cells, the

342 latter probably reflecting recognition of processed intact aggrecan known to be
343 released by degrading cartilage (52-54). The data suggest that type B T cell ligands
344 are less abundant in degrading cartilage than intact aggrecan, and are more likely to
345 be rapidly degraded. It has also been shown that capture and presentation of
346 extracellular peptide epitopes by surface MHC class II is inefficient (45,59) but DM
347 independent (47,48), and so would be permissive for type B T cell recognition.
348 Type B T cells recognising exogenous self antigens have been suggested to persist
349 in the periphery due to escape from negative selection during thymic development,
350 circumstances in which type A T cells would be largely deleted. Consequently we
351 propose that type B T cells recognizing cartilage antigens may represent a significant
352 and even dominant contribution to the T cell response in autoimmune arthritis (15, 20).
353 Increased turnover of cartilage due to chronic trauma or reactive inflammation caused
354 by infection could release peptides or fragments from cartilage autoantigens above a
355 threshold required for type B T cell activation. However, it remains to be determined
356 whether type B T cells perform regulatory functions, provide help for pathogenic
357 autoantibody responses, or differentiate into IFN-γ and IL-17 producing effector T cells
358 implicated in joint damage in the animal models of arthritis, in RA in humans, and in
359 other automimmune diseases.

Acknowledgements

This research was funded by a Nuffield Foundation Oliver Bird Rheumatism Programme Studentship to JF, and by grants from Arthritis Research UK and The JGW Patterson Foundation.

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Figure legends

Figure 1. Lymph node responses to aggrecan and peptide. BALB/c mice were immunized with 25 µg aggrecan (a), or 100 µg peptide (b) in adjuvant, and 7 days later proliferation of PLN cells was assayed by tritiated thymidine incorporation in response to a dose range of peptide (circles) or aggrecan (triangles). Proliferation of PLN from HBSS-immunized mice was used as a negative control (open symbols in a), and responses of PLN to 5 µg/ml concanavalin A (con A) as a positive control (closed squares). Results are expressed as mean cpm ± SEM of 5 mice.

Figure 2. **T cell hybridoma responses to peptide and aggrecan.** 4 x 10^4 per well of T cell hybridomas 2G9 (a,e,i), 1271b (b,f,j), 1G2 (c,g,k) or 2D7 (d,h,l) were incubated for 24 h with 4 x 10^4 per well of either bone marrow derived macrophages, bone marrow-derived DC, or the B lymphoma line A20 as APC, and a range of doses of aggrecan or aggrecan peptide 84-103. Responses were measured as cytokine release into the supernatant in a bioassay with IL-2 dependent CTLL-2 cells as described in Materials and Methods, and results are shown as cpm ± SEM for triplicate wells for a representative of at least two different experiments for each hybridoma.

Figure 3. Fine mapping T cell hybridoma responses to peptide. T cell hybridomas 2G9, 1271b, 1G2 and 2D7 were assayed for responses to 10 µM doses of the staggered (row A) or alanine substituted (row B) peptides shown, using bone marrow derived macrophages as APC as in the legend to Fig. 2, and results are shown as cpm ± SEM for triplicate wells. Controls were T cells and APC without peptide (0 in A and B), and 200 nM aggrecan (aggrecan in A), or 10 µM unsubstituted peptide (89-103 in

B).

Figure 4. Hybridoma responses to citrullinated peptide 84-103. T cell hybridomas 2G9 (a), 1271b (b), 1G2 (c) or 2D7 (d) were assayed as described in the legend to Fig. 2, except that 4 x 10^4 per well of the macrophage cell line J774A.1 were added as APC, and the antigen for the dose titrations were the native aggrecan peptide 84-103 (93R95R), or the same peptide with citrulline substituted for the arginine at position 93 (93cit95R), position 95 (93R95cit), or both (93cit95cit).

Figure 5. T cell hybridoma responses to supernatants from degrading cartilage explants. T cell hybridomas 1271b (a,d), 1G2 (b,e) and 2D7 (c,f) were assayed for responses to (column A) supernatants collected from bovine cartilage explants after 1, 5, 10 15 and 20 days of culture (showing one experiment of three), or (column B) supernatants collected from human articular cartilage explants after days 15 days of culture (showing supernatants from three different experiments HAC1-3). Preparation of supernatants is described in Materials and Methods, and supernatants were titrated and shown as percent of the total culture volume (the average protein concentration 3.07 mg/ml for human and 1.5 mg/ml for bovine cartilage explant supernatants, of which the majority is known to be aggrecan (30)). Bone marrow derived macrophages were used as APC and results are shown as cpm ± SEM for triplicate wells. Controls included 1 μM peptide 84-103 (84-103) and absence of peptide (0).

624 Figure 1. Lymph node responses to peptide 84-103 and aggrecan

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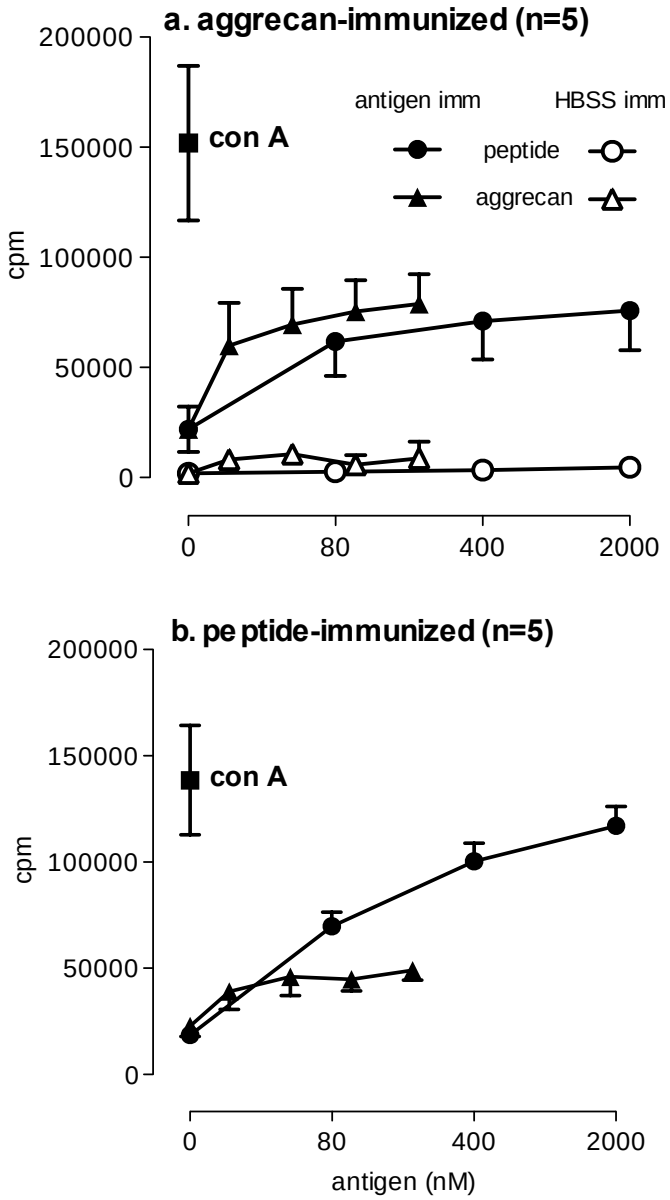

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628 Figure 2. T cell hybridoma responses to peptide 84-103 and aggrecan

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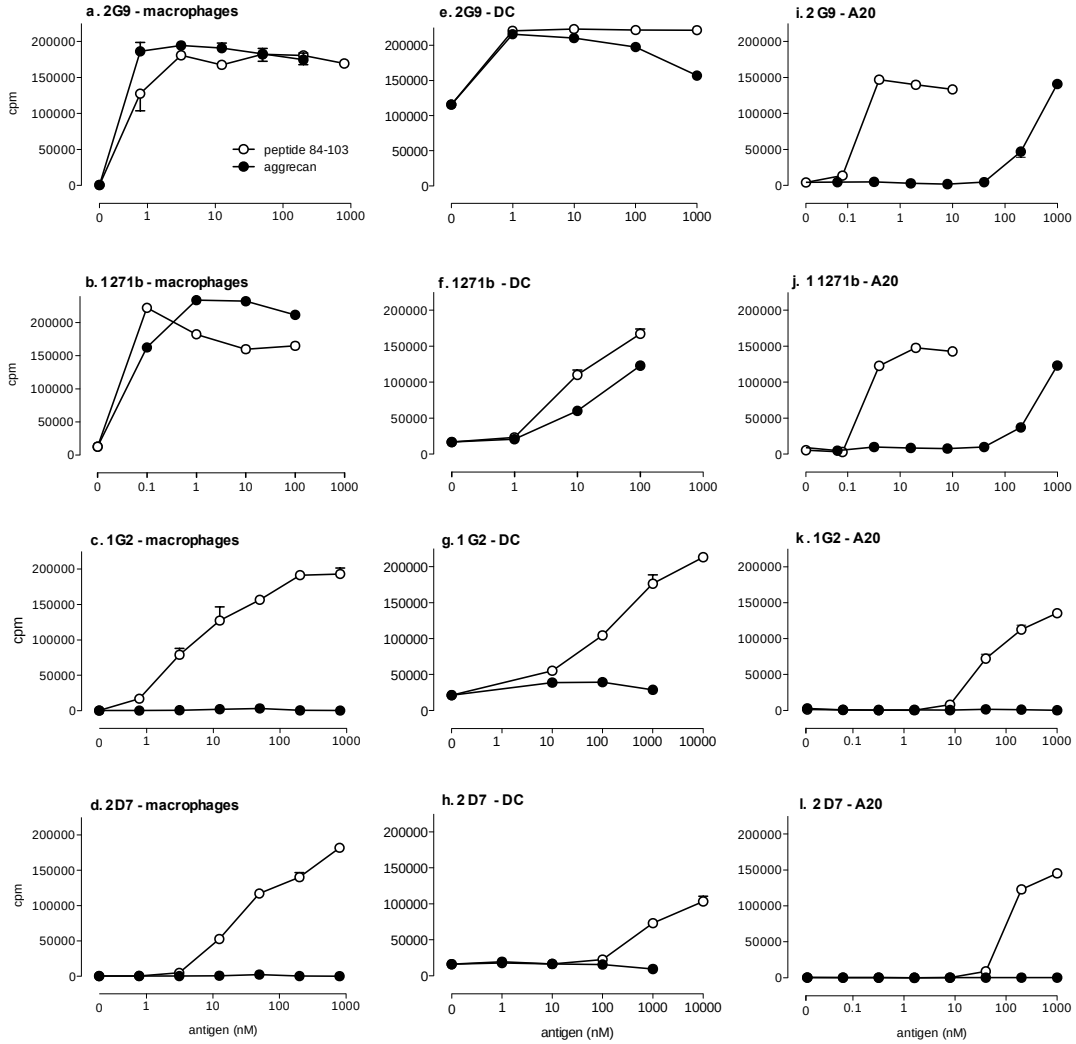

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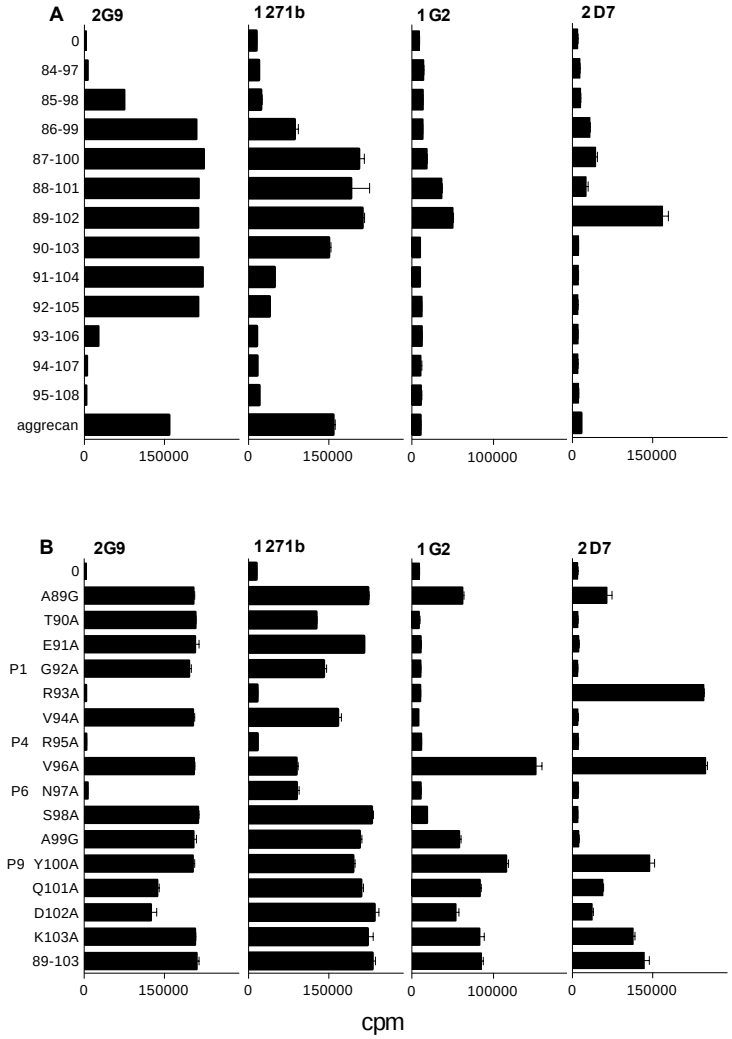
Figure 3. Fine mapping T cell hybridoma responses to peptide 84-103.



639 Figure 4. Hybridoma responses to citrullinated peptides.

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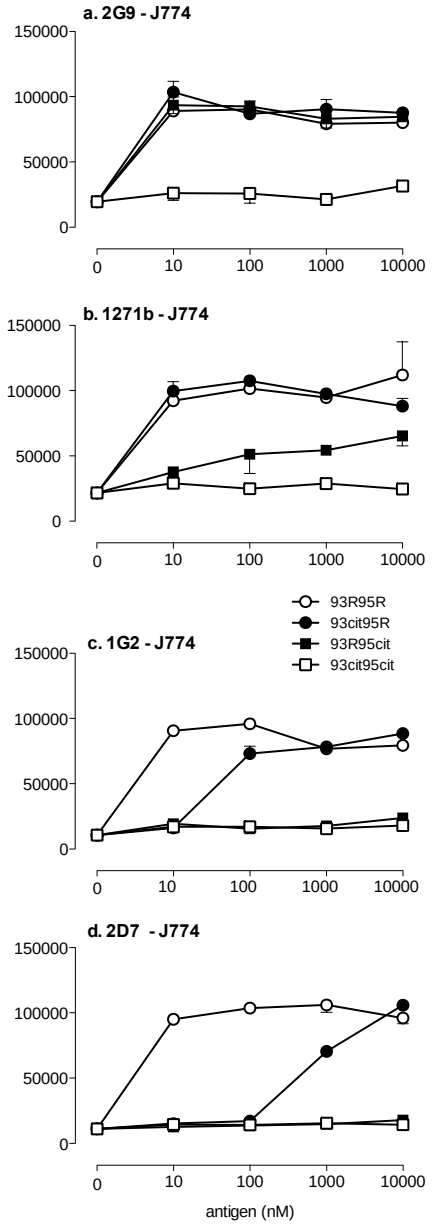

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643 Figure 5. T cell hybridoma responses to supernatants from cartilage explants

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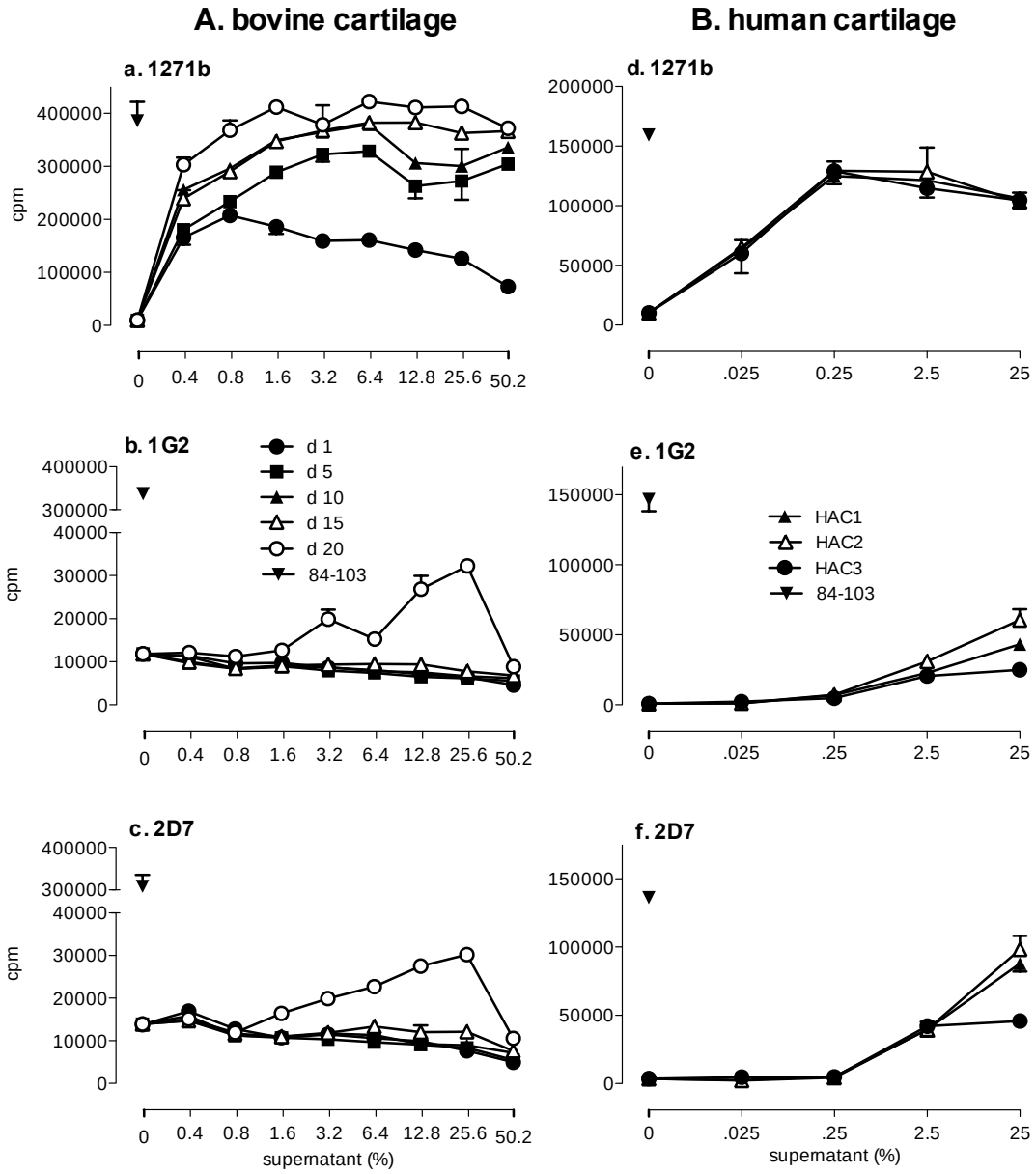

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